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Downregulation of PPP2R5E expression by miR-23a suppresses apoptosis to facilitate the growth of gastric cancer cells

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ABSTRACT

PPP2R5E belongs to the phosphatase 2A regulatory subunit B family and acts as a tumor suppressor in human cancer. However, the role of PPP2R5E in the tumorigenesis of gastric cancer is unclear. Here, we declare that PPP2R5E is downregulated by miR-23a and induces cell growth inhibition and apoptosis in gastric cancer cells. Furthermore, ASO-miR-23a suppresses tumor growth derived from MGC803 cells in vivo. PPP2R5E is identified as a new target of miR-23a. Moreover, overexpression of PPP2R5E reversed the negative effects of miR-23a. We highlight the significance of miR-23a and PPP2R5E in the proliferation and apoptosis of gastric cancer cells.

1. Introduction

Gastric cancer, which ranks as the second leading cause of cancer worldwide, has a poor prognosis and a high mortality rate [1–3]. Previous reports have demonstrated that gastric cancer is a disease involving multiple factors and steps [4–6]. Accumulated evidence has shown that the pathogenesis of gastric cancer involves the activity of oncogenes or tumor suppressors [7,8]. However, the molecular mechanisms underlying the development and progression of gastric cancer remain unknown. Serine/threonine-protein phosphatase 2A 56-kDa regulatory subunit epsilon isoform (PPP2R5E) belongs to the phosphatase 2A (PP2A) regulatory subunit B family and acts as a tumor suppressor in human cancers. Recent studies have reported that the presence of polymorphisms in PP2A subunit genes, including the PPP2R5E gene, increase the risk for lung cancer [9]. However, the role of PPP2R5E in the development of gastric cancer is unknown.

miRNAs are a group of evolutionarily conserved, small non-coding single-stranded RNAs that can regulate gene expression by binding to specific regions of target mRNAs to induce mRNA degradation or to suppress mRNA translation [10–12]. miRNAs participate in different physiological processes, depending on their target gene [13,14]. Some miRNAs can function as oncogenes and participate in tumorigenesis [15]. miR-19a/b has been shown to facilitate the migration, invasion and metastasis of gastric cancer cell in vitro and in vivo [16], and miR-23a and miR-27a were shown to be highly expressed in gastric cancer cells and to contribute to their growth [17–19]. In contrast, other miRNAs have been shown to function as tumor suppressor genes and to be expressed at a low level in tumor tissues. The expression of the miR-200bc/429 cluster, miR-497 and miR-181b, which negatively regulate the expression of BCL2 and contribute to the apoptosis-mediated multidrug resistance, is downregulated in gastric cancer cell lines [20–22].

Recently, miR-23a was found to perform various biological functions in different types of tumors; in gastric tumors, miR-23a was found to target MT2A and to promote tumor growth [23]. The Fas signaling pathway has been shown to induce the epithelial–mesenchymal transition (EMT) to promote the metastasis of gastrointestinal cancer cells, but the involvement of miRNAs in this mechanism is unknown. Zheng found that treatment with the Fas ligand inhibited the expression of E-cadherin and promoted cell invasion by upregulating the expression of miR-23a, whereas the overexpression of an miR-23a inhibitor partially blocked this

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activity [24]. The miR-23a cluster is a downstream target of PU.1 and is involved in antagonizing the lymphoid cell fate [25]. Yong found that miR-23a appeared to be a potential blood biomarker for the early detection of colorectal cancer [26]. miR-23a promoted the metastasis of neuroblastoma cells through inhibiting the expression of the CDH1 gene [27] and targeted glutaminase (GLS) mRNA and inhibited the expression of GLS protein in human leukemic cells [28]. The miR-23a/24/27a cluster promoted cellular proliferation and suppressed apoptosis in liver cancer cells [29]. Compared with the level in normal bladder mucosa, the level of expression of miR-23a was significantly upregulated in bladder cancers [30].

In the current study, we found that PPP2R5E promoted apoptosis to suppress the growth of cells of gastric cancer cell lines and its expression was downregulated in human gastric cancer tissues. Furthermore, miR-23a was identified as a regulator of PPP2R5E expression. The suppression of PPP2R5E expression through binding the 3′ UTR of miR-23a promoted the growth and suppressed the apoptosis of gastric cancer cells and promoted the growth of tumors derived from MGC803 cells in vitro and in vivo. Thus, a decrease in the level of PPP2R5E expression and a concomitant increase in the level of miR-23a expression in gastric cancer tissues appears to promote gastric carcinogenesis.

2. Materials and methods

2.1. Human cancer tissue specimens and RNA isolation

Freshly frozen human gastric cancer tissue and matched non-tumor gastric tissue specimens were obtained from the Tumor Bank Facility of Tianjin Medical University Cancer Institute and Hospital and from the National Foundation for Cancer Research. The subtype of each tumor was verified by histological analysis. The use of human materials conformed to the policies of our Institutional review board. The total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion, Austin, TX). Large RNAs (those larger than 200 nt) and small RNAs (those smaller than 200 nt) were separated and purified following the manufacturer's instructions.

2.2. Quantitative reverse transcription–PCR (qRT-PCR)

qRT-PCR was performed to detect the levels of miR-23a and protein phosphatase 2 regulatory subunit B′ (PPP2R5E) mRNAs in the tissue specimens. The small RNAs that were extracted from the tissue were reverse transcribed into cDNA. The cDNA was used to amplify mature miR-23a transcripts and the endogenous control, U6 snRNA, using PCR. To quantify PPP2R5E gene expression, 5 μg of RNA extracted from cells or tissue specimens was reverse transcribed to cDNA using M-MLV reverse transcriptase. PCR amplification was performed using the SYBR Premix Ex Taq™ kit (TaKaRa, Otsu, Shiga, Japan), and qRT-PCR was performed using an iQ5 qRT-PCR detection system (Bio-Rad, Hercules, CA). The relative gene expression levels were calculated using the $2^{-\Delta\Delta ct}$ method [31]. All of the primers were purchased from AuGCT, Inc. (Beijing, China), and the sequences of the primers are shown in Table 1.

2.3. Immunohistochemistry

Immunohistochemistry was performed according to previously described methods [32]. The sections were pretreated using microwave irradiation, then were blocked and incubated with polyclonal rabbit anti-human PPP2R5E antibodies (Saier Biotechnology). The staining intensity was then assessed.

2.4. Plasmid construction

The pSilencer/shRNA-PPP2R5E (sh-PPP2R5E) vector was obtained by annealing two single-stranded complementary sequences that contained BamHI and HindIII restriction sites at the ends. The fragment was then cloned into the pSilencer2.1/neo vector (Ambion) between the BamHI and HindIII sites. A 1400-bp fragment was inserted into the pCD3/3flag vector, which contained BamHI and XhoI restriction sites. The pcDNA3 vector was used to generate a PPP2R5E-overexpression plasmid. The full-length human PPP2R5E cDNA sequence (GenBank TM, NM_017423.2) was amplified by PCR using cDNA isolated from fetal brain tissue as the template. The PPP2R5E gene was inserted into the EcoRI and XbaI restriction sites. The construction of the

Table 1
The oligonucleotides used in this work.

Name	Sequence (5′–3′)
pri-miR-23a forward	5′-GCGAGATCTGGCTCCTGCATATGAG-3′
pri-miR-23a reverse	5′-GATGAATTCCAGGCACAGGCTTCGG-3′
ASO-miR-23a	5′-GGAAATCCCTGGCAATGTGAT-3′
ASO-NC	5′-GTGGATATTGTTGCCATCA-3′
PPP2R5E-3′ UTR-S	5′-CGCGGATCCAAATTCCATTATCGGGAG-3′
PPP2R5E-3′ UTR-A	5′-CGGGATTCTCCAGAGGAGGATGTTACAC-3′
PPP2R5E-3′ UTR-MS	5′-GATACAATTTTCATAAGAGTACAATCTTAAATTTAGC-3′
PPP2R5E-3′ UTR-MA	5′-GCTAAATTTAAGATTGTACTCTTATGAAAATTGTATC-3′
PPP2R5E-qPCR-AS	5′ GTAGCATTTGTAGAACCAC 3′
PPP2R5E-qPCR-S:	5′ TTGGAGTTGTTGCCTTAC 3′
PPP2R5E-S-BamHI	5′ GCGGATCCAAAGTAGGGATATG 3′
PPP2R5E-AS-XhoI	5′ CCCCTCGAGTTAGATGTTGTTGTCATT 3′
PPP2R5E-siR-Top	5′-GATCCGCAGAAGAAGATGAACCTACTTC AAGAGAGTAGGTTCATCTTCTTCTGTTTTTT GGAAA-3′
PPP2R5E-siR-Bot	5′-AGCTTTTCCAAAAAACAGAAGAAGAT GAACCTACTCTCTTGAAGTAGGTTCATCTTC TTCTGCG-3′
β-Actin-S	5′-CGTGACATTAAGGAGAAGCTG-3′
β-Actin-A	5′-CTAGAAGCATTTGCGGTGGAC-3′
miR-23a-RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACGGAAATCC-3′
U6 RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAATATGG-3′
miR-23a forward	5′-TGCGGATCACATTGCCAGG-3′
U6 forward	5′-TGCGGGTGCTCGCTTCGGCAGC-3′
Reverse	5′-CCAGTGCAGGGTCCGAGGT-3′
GAPDH-S	5′-GCGAATTCCGTGTCCCCACTGCCAACGTGTC-3′
GAPDH-AS	5′-GCTACTCGAGTTACTCCTTGGAGGCCATGTGG-3′

pcDNA3/pri-miR-23a plasmid was described In our previously published paper [33]. We also had a 2′-O-methyl-modified antisense oligonucleotide directed against miR-23a (ASO-miR-23a) synthesized commercially (GenePharm, Shanghai, China). The sequence of the ASO-miR-23a construct that was used in this study is shown in Table 1. The enhanced green fluorescent protein (EGFP) expression vector (pcDNA3/EGFP) was constructed as previously described. The 3′ UTR fragment of the PPP2R5E gene containing the predicted miR-23a binding site was amplified by PCR using the primers. The PCR products were cloned into the pcDNA3/EGFP plasmid between the BamHI and EcoRI restriction sites, and the resulting vector was named pcDNA3/EGFP-PPP2R5E 3′ UTR. Moreover, a mutant fragment of the PPP2R5E 3′ UTR, which contained a mutated miR-23a binding site, was amplified by PCR site-directed mutagenesis and cloned into the pcDNA3/EGFP plasmid between the BamHI and EcoRI restriction sites (pcDNA3/EGFP-PPP2R5E 3′ UTR-mut). All of the inserted DNAs mentioned above were verified by DNA sequencing. The primers are shown in Table 1.

2.5. Western blotting analysis

At 72 h after transfection, the cells were collected and lysed using RIPA lysis buffer (0.15 M NaCl, 0.05 M Tris HCl, pH 7.2, 1% Triton X-100 and 0.1% SDS). The protein concentration was determined using the BCA reagent purchased from Promega. Approximately 25 μg of each protein sample was subjected to SDS–PAGE and then transferred to a nitrocellulose membrane. The membranes were blocked using 5% blotto for 2 h at room temperature, followed by an overnight incubation at 4 °C with anti-PPP2R5E (1:200) and anti-tubulin (1:1000) antibodies, and then were incubated with HRP-conjugated secondary antibody (1:1000). The bands were detected using an enhanced chemiluminescence reagent and the membranes were exposed to autoradiographic film, and then the band intensities were quantitated using Analysis Software. The antibodies were obtained from Saier Biotechnology.

2.6. Cell culture and transfection

The MGC803 and BGC823 human gastric cancer cell lines were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were grown in RPMI1640 medium (GIBCO BRL, Grand Island, NY, USA) containing with 10% fetal bovine serum (FBS), 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin. These two cell lines express high levels of miR-23a, which remained stable during culturing, passaging and transfection in our laboratory; therefore, we chose these two cell lines for the functional studies.

One day before transfection, the cells were plated in a 48-well plate at approximately 2×10^4 cells/well. The transfections were performed using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The oligonucleotides and plasmids were used at a final concentration of 100 nM and 5 ng/μL, respectively. To determine the transfection efficiency, we transfected either pcDNA3-EGFP or the negative control, pcDNA3, into the two cell lines and assessed the level of EGFP expression 48 h after transfection using fluorescence microscopy. As expected, the efficiency of transfection was approximately 60–70%.

2.7. MTT and colony-formation assays

The cells were seeded in a 96-well plate at 4×10^3 per well and were transfected with 100 nM ASO-miR-23a or with 5 ng/μL of each plasmid, as described above. At 48 and 72 h post-transfection, the cells were incubated with 10 μL of MTT for 4 h and then were dissolved in 100 μL of DMSO. After shaking for 10 min, the absor-

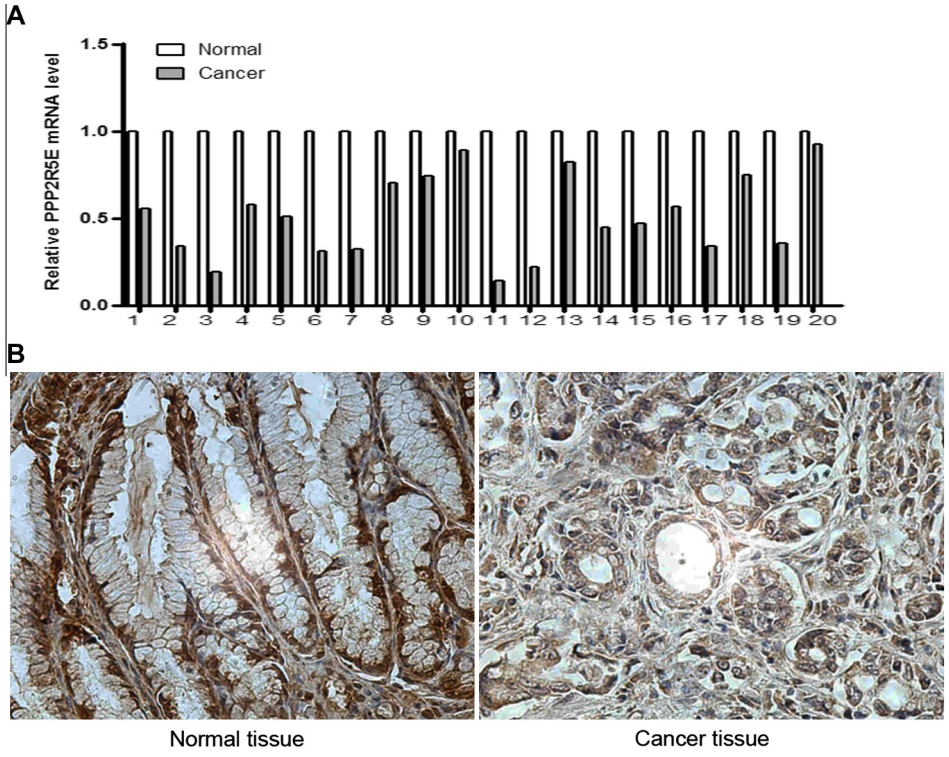

Fig. 1. The level of PPP2R5E expression in gastric cancer tissues. (A) The relative level of PPP2R5E mRNA in 20 pairs of gastric cancer tissues was evaluated using qRT-PCR. β-actin was included as an endogenous control ($n = 20$, $P < 0.05$). (B) The level of expression of PPP2R5E in gastric cancer tissues and in matched normal tissues was assessed using immunohistochemistry ($n = 20$, $P < 0.05$).

bance at 570 nm was measured using an lQuant Universal Microplate Spectrophotometer (Bio-tek Instruments).

After transfection, the cells were plated in 12-well plates at 300 BGC823 cells or 200 MGC803 cells per well for the colony-formation assay. During colony growth, the culture medium was replaced every 3–5 days. The number of MGC803 and BGC823 colonies was counted on the 10th day and 14th day, respectively, after crystal-violet staining.

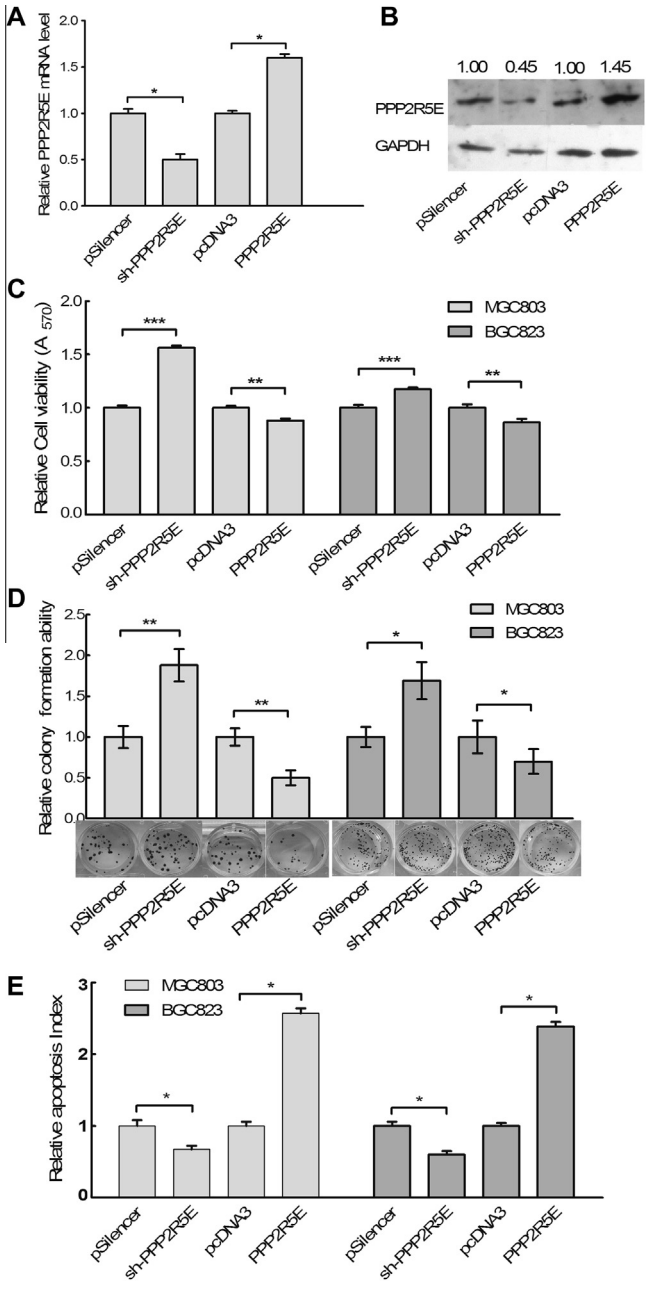

Fig. 2. Knocked-down expression or overexpression of PPP2R5E altered the phenotypes of gastric cancer cells. (A) Cells of the MGC803 cell line were transfected with pSilencer/sh-PPP2R5E, pcDNA3/3flag-PPP2R5E or the appropriate control vectors, and qRT-PCR was performed to detect the level of PPP2R5E expression ($n = 3$, $^{*}P < 0.05$). (B) The PPP2R5E protein expression level in transfected MGC803 cells was evaluated using western blotting analysis. The Arabic numerals above the western blot image indicate the ratio of the densitometric values of PPP2R5E and GAPDH compared with those of the control. (C) Cells of the MGC803 and BGC823 cell lines were transfected with pSilencer/sh-PPP2R5E, and the growth and viability of the two cell lines were evaluated using the MTT assay ($n = 3$, $^{**}P < 0.01$, $^{***}P < 0.001$). (D) Cell clonogenicity was evaluated using a colony-formation assay. MGC803 cells were grown for 10 days, and BGC823 cells were grown for 14 days. The images demonstrate the stained colonies ($n = 3$,$^{*}P < 0.05$, $^{**}P < 0.01$). (E) Paclitaxel (0.05 ppc) was added to the transfected cells to induce apoptosis. A TUNEL assay was performed to detect apoptotic cells. The induced-apoptosis indices are shown ($n = 3$, $^{*}P < 0.05$).

2.8. TUNEL assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using the In-Situ Cell Death Detection Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). At 24 h after transfection, the cells were seeded at 5×10^3 cells per well in 14-well plates. Then, the cells were treated with 0.05 ppc paclitaxel for 1 h and fixed using 4% paraformaldehyde. Next, the cells were incubated in 0.1% TritonX-100 for 2 min on ice. After washing the cells, 4 μL of the TUNEL reaction mixture was added to each well, and cells were incubated for 1 h at 37 °C the dark. Finally, DAPI (4,6-diamidino-2-phenylindole, Dojindo Molecular Technologies, Inc., Japan) was used to stain the nuclei. Fluorescence images were obtained at an excitation wavelength of 488 nm for FITC-labeled nanoparticles using NIS Elements F 2.20 imaging software (Nikon, Tokyo, Japan).

2.9. Fluorescent reporter assay

MGC803 cells were transfected in 48-well plates with the PPP2R5E EGFP reporter vector and with the wild-type or a mutated 3′ UTR vector and were also co-transfected with miR-23a ASO or pcDNA3/pri-23a. The RFP expression vector pDsRed2-N1 was transfected at 0.05 μg per well (Clontech, Mountain View, CA, USA). At 48 h after transfection, the cells were lysed using RIPA buffer. The fluorescence intensities of EGFP and red fluorescent protein were measured using a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

2.10. Murine xenograft model

Six-week-old female nude mice were purchased from the animal facilities of the Chinese Academy of Medical Sciences and were housed in the animal facilities of Tianjin Medical University as approved by the Institutional Animal Care and Use Committee. MGC803 cells (4×10^6 cells/0.15 mL/mouse) were subcutaneously injected into the flanks of the nude mice. After 3 weeks, the mouse were sacrificed, the tumors were harvested and measured, then they were stored at −80 °C for subsequent analysis.

2.11. Bioinformatic methods and statistical analysis

Bioinformatic methods were used to predict the miRNA targets of miR-23a. we chose two commonly utilized algorithms, TargetScan Release 4.0 (http://www.targetscan.org) and PicTar (http://pictar.bio.nyu.edu/cgi-bin/PicTar_vertebrate.cgi). PPP2R5E was predicted as a candidate target gene of miR-23a.

Each experiment was performed in triplicate. The differences between multiple groups were analyzed using an ANOVA, and the significance of the differences between independent groups were evaluated using the paired Student's t test, with P set at <0.05.

3. Results

3.1. PPP2R5E expression is suppressed in gastric cancer cells

We first performed qRT-PCR to examine the differential expression of PPP2R5E in 20 pairs of gastric cancer tissues and the adja-

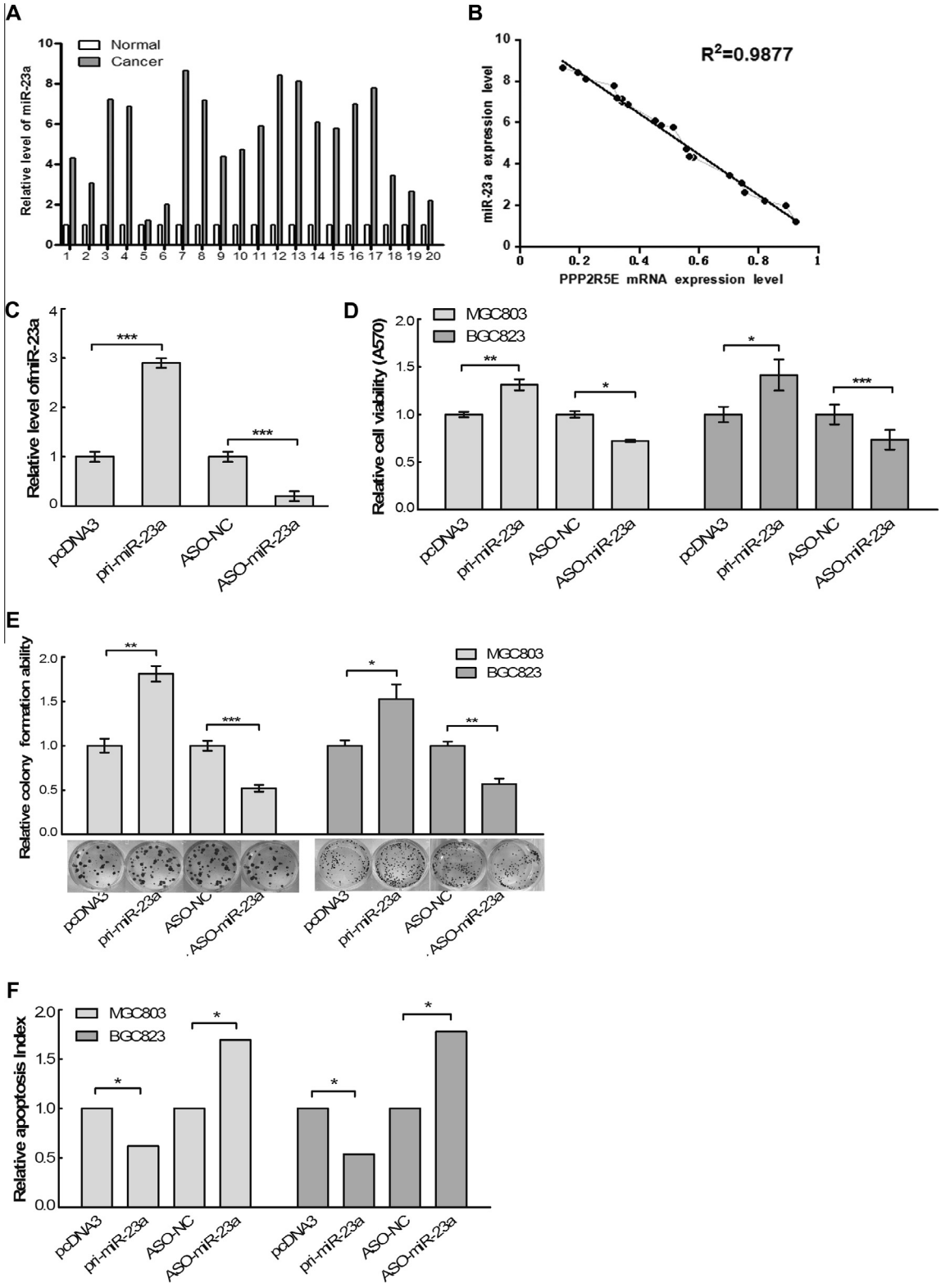

Fig. 3. The effects of overexpression or knocking down the expression of miR-23a on MGC803 and BGC823 cells. (A) qRT-PCR was used to examine miR-23a expression in 20 pairs of gastric cancer tissues and their corresponding adjacent normal tissues. U6 RNA was included as an endogenous housekeeping gene, and the relative level of miR-23a expression is shown (n =20, $P < 0.05$). (B) The Spearman correlation test was performed to analyze the inverse relationship between PPP2R5E and miR-23a expression in gastric tissues ($R^2 = 0.9877$). (C) miR-23a levels in MGC803 cells transfected with pcDNA3, pri-miR-23a, ASO-NC or ASO-miR-23a. U6 RNA was included as an endogenous housekeeping gene ($n = 3$, $^{***}P < 0.001$). (D) The MTT assay was used to analyze the cell viability of MGC803 and BGC823 cells transfected with pcDNA3, pri-miR-23a, ASO-NC or ASO-miR-23a 72 h after transfection ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (E) The colony-formation rate of MGC803 and BGC823 cells transfected with pcDNA3, pri-miR-23a, ASO-NC or ASO-miR-23a. Representative images of stained colonies are shown ($n = 3$,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (F) A TUNEL assay was used to evaluate apoptosis among MGC803 and BGC823 cells transfected with pcDNA3, pri-miR-23a, ASO-NC or ASO-miR-23a and exposed to Paclitaxel (0.05 ppc). The induced-apoptosis indices are shown ($n = 3$, $^{*}P < 0.05$).

cent, non-cancerous tissues. As shown in Fig. 1A, the results showed that the expression of PPP2R5E mRNA was downregulated in the 20 gastric cancer tissue specimens. To confirm the reduced levels of PPP2R5E expression in these tissues, we utilized immunohistochemical staining to detect the PPP2R5E protein levels. Compared with those of the normal gastric tissues, the levels of PPP2R5E protein expression were markedly decreased in the gastric cancer specimens (Fig. 1B). These observations led us to expect that the level of expression of PPP2R5E was repressed in gastric cancer cells.

3.2. PPP2R5E suppressed the growth of and induced apoptosis in gastric cancer cells

Previous studies have demonstrated that PPP2R5E played an important role in suppressing tumor-cell proliferation and displayed tumor-suppressive properties in breast cancer [34]. To determine its role in gastric cancer cells, we first constructed a pSilencer/sh-PPP2R5E plasmid to block the endogenous expression of PPP2R5E, and a pCD3/3flag-PPP2R5E vector that lacked the its 3′ UTR. The expression of PPP2R5E in transfected MGC803 cells was determined using qRT-PCR and western blotting analysis. As shown in Fig. 2A and B, pSilencer/sh-PPP2R5E effectively suppressed and pCD3/3flag-PPP2R5E effectively increased the levels of PPP2R5E mRNA and protein expression.

To evaluate the effect of PPP2R5E on cell viability, we performed an MTT assay. Compared to that of the control groups, knocking down PPP2R5E expression increased, whereas overexpression of PPP2R5E decreased, the viability of MGC803 and BGC823 cells (Fig. 2C). After enhancing or blocking the expression of PPP2R5E, the long-term and independent cellular growth ability of these cells was evaluated using a colony-formation assay. Consistent with the results of MTT assay, the inhibition of PPP2R5E expression led to increased colony-formation ability, whereas the overexpression of PPP2R5E led to a reduction of that ability in MGC803 and BGC823 cells (Fig. 2D). Next, the TUNEL assay was used to evaluate apoptosis in the gastric cancer cell lines. Knocking down PPP2R5E expression decreased the relative apoptosis index to 67% and 60% in MGC803 and BGC823 cells, respectively (Fig. 2E). The overexpression of PPP2R5E increased the relative apoptosis index by approximately 2.57-fold and 2.39-fold in MGC803 and BGC823 cells, respectively (Fig. 2E), compared with those of the negative control groups. Representative images showing the apoptotic cells are shown in Fig. S1.

Together, these observations indicated that PPP2R5E repressed cell viability and growth and promoted apoptosis in cells of the gastric cancer cell lines, which provided evidence and support for its tumor-suppressive role in gastric cancer.

3.3. miR-23a functions as an oncogene in gastric cancer cells

To explore the mechanism for the downregulation of PPP2R5E expression in gastric cancer cells, bioinformatic analysis was utilized to predict miRNAs that might serve as post-transcriptional regulators of PPP2R5E. We found many miRNAs that could potentially bind to the PPP2R5E 3′ UTR, including miR-23a, miR-19, miR-181 and others. Moreover, our previous study had revealed that miR-23a expression was clearly upregulated in gastric cancer cells [33]. Therefore, miR-23a was chosen for further investigation in this study.

qRT-PCR was applied to detect the level of miR-23a expression in another 10 pairs of gastric cancer tissue and matched normal gastric tissue specimens to confirm the upregulation of miR-23a expression in gastric cancer tissue (Fig. 3A). We utilized the Spearman correlation to analyze the relationship between the expression PPP2R5E and miR-23a in the gastric cancer tissues. As shown in Fig. 3B, the levels of miR-23a and PPP2R5E expression had a strong inverse relationship ($R^2 = 0.9877$). Human gastric cancer cells were transfected with the pri-miR-23a vector, ASO-miR-23a and their respective controls, and the efficiency of the effect of pri-miR-23a or ASO-miR-23a in BGC823 cells was validated using qRT-PCR. We confirmed that pri-miR-23a increased the level of miR-23a expression and that ASO-miR-23a effectively decreased it (Fig. 3C). The results of the MTT assay showed that cell viability increased approximately 1.31-fold in MGC803 cells and 1.41-fold in BGC823 cells when miR-23a was overexpressed. Conversely, ASO-miR-23a reduced the viability of MGC803 cells to 72% and that of BGC823 cells to 74% (Fig. 3D). The results of the colony-formation assay revealed a marked reduction in the colony-formation rate of MGC803 and BGC823 cells that were treated with ASO-miR-23a relative to that of the corresponding negative controls.

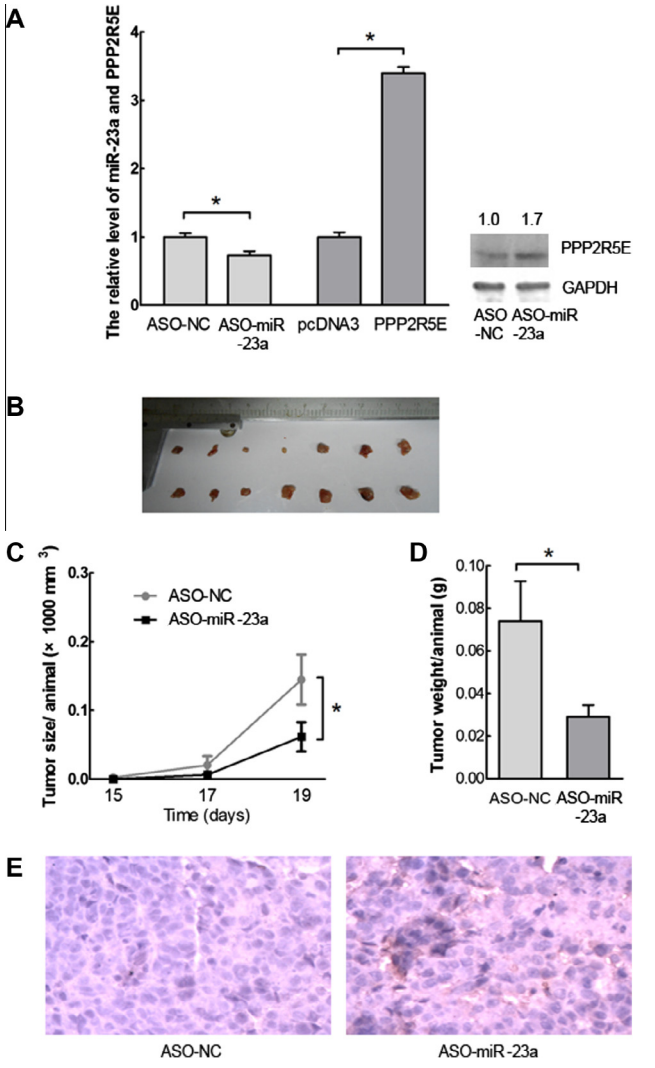

Fig. 4. ASO-miR-23a suppressed the growth of tumors derived from MGC803 cells in nude mice. (A) The cancer cells were injected subcutaneously into two groups of nude mice, and nodules were obtained from the 14 mice that were injected with MGC803 cells transfected with ASO-miR-23a or ASO-NC ($n = 7$, $^*P < 0.05$). (B) The cancer cells were injected subcutaneously into two groups of nude mice, and the size of the tumors was monitored at different time points. The mean size of the tumors per animal was plotted ($n = 7$, $^*P < 0.05$). (C) The average weight of the tumor nodules derived from MGC803 cells that were transfected with ASO-NC or ASO-miR-23a in SCID mice ($n = 7$, $^*P < 0.05$). (D) qRT-PCR and western blotting assay were performed to evaluate the levels of miR-23a and PPP2R5E expression in the tumor nodules. (E) The expression of PPP2R5E in the tumor nodules of the ASO-NC and ASO-miR-23a groups was detected using immunohistochemistry ($n = 7$, $P < 0.05$).

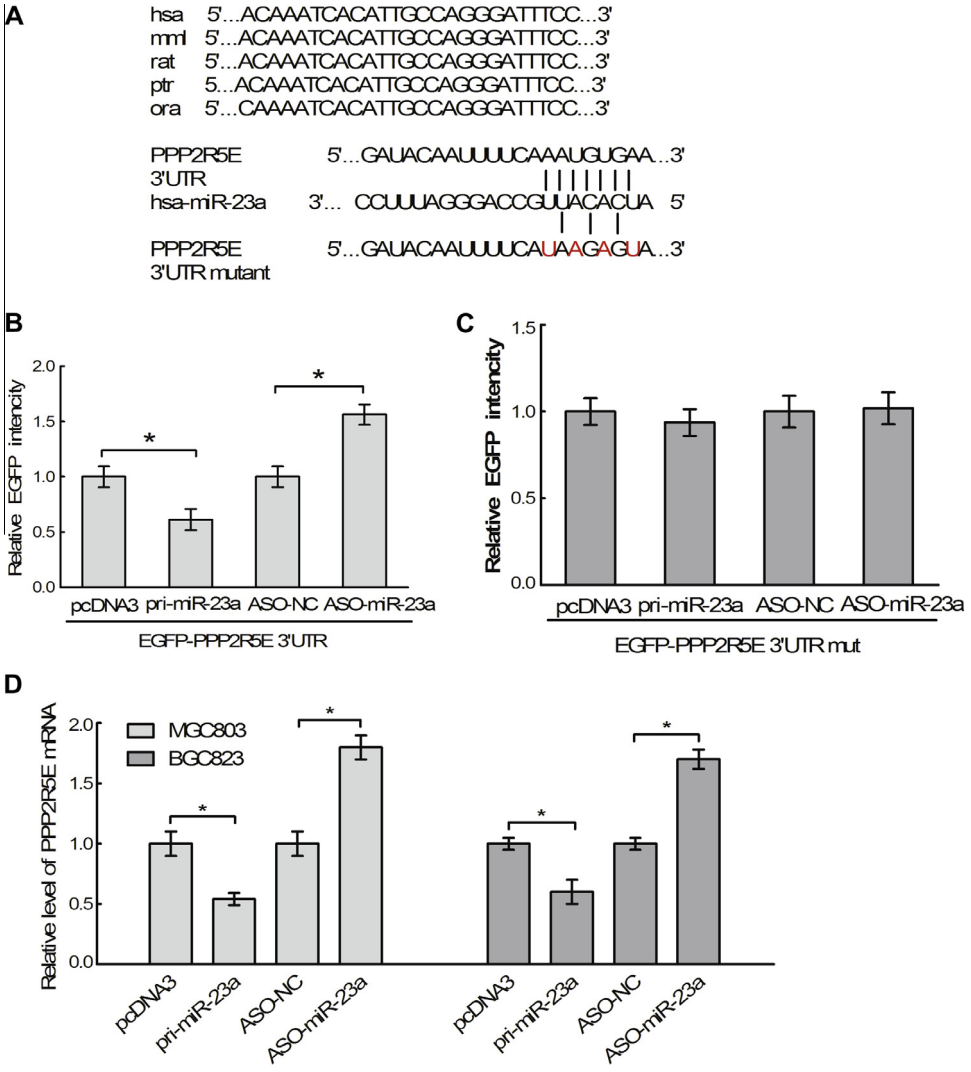

Fig. 5. PPP2R5E is a direct target of miR-23a. (A) As predicted by the Target Scan and PicTar algorithms, the PPP2R5E 3′ UTR has a miR-23a-binding site. The sequences of the mutated PPP2R5E 3′ UTR, in which several nucleotides within the miR-23a binding site were mutated, is shown. (B) The direct interaction between miR-23a and the PPP2R5E 3′ UTR was confirmed using an EGFP-reporter assay. MGC803 cells were transfected with the EGFP-PPP2R5E 3′ UTR reporter gene and with pcDNA3, pri-miR-23a, ASO-NC or ASO-miR-23a ($n = 3$, $^*P < 0.05$). (C) The fluorescence intensity of MGC803 cells that were transfected with the EGFP vector, the PPP2R5E 3′ UTR reporter or the mutant EGFP-3′ UTR reporter in addition to pcDNA3, pcDNA3/pri-miR-23a, ASO-NC or ASO-miR-23a. (D) The level of expression of PPP2R5E mRNA in MGC803 and BGC823 cells that were transfected with pcDNA3, pcDNA3/pri-miR-23a, ASO-NC or ASO-2miR-3a was determined using qRT-PCR ($n = 3$, $^*P < 0.05$).

Conversely, the overexpression of miR-23a significantly increased the colony-formation ability of these two human gastric cancer cell lines (Fig. 3D and E). To assess whether miR-23a had an effect on cellular apoptosis, we performed the TUNEL assay in parallel. Compared with those of the negative control groups, the apoptosis index was approximately 62% or 54% lower in the pri-miR-23a groups and 1.69 or 1.78-fold higher in the ASO-miR-23a groups of MGC803 or BGC823 cells, respectively (Fig. 3E and F). Moreover, overexpression of miR-23a resulted in a significantly reduced rate of apoptosis of MGC803 and BGC823 cells, and the opposite result was obtained in cells that were transfected with ASO-miR-23a (Fig. S2). Collectively, these observations strongly suggested that miR-23a is apparently capable of suppressing apoptosis and promoting the growth, viability and colony-formation ability of MGC803 and BGC823 cells.

To examine the effect of miR-23a on the growth of tumors in vivo, we transfected MGC803 cells with ASO-miR-23a or ASO-NC and injected the transfected cells into the flanks of 7 nude mice. The tumor nodules recovered after sacrificing the mice are shown in Fig. 4A. The average size (Fig. 4B) and weight (Fig. 4C) of the tumors in the ASO-miR-23a group were less than those of the ASO-NC group. We also examined the level of miR-23a and PPP2R5E expression in the tumor nodules. The results showed that the miR-23a level of the tumor nodules from the ASO-miR-23a group was decreased to 27% but their levels of PPP2R5E mRNA and protein were increased 3.4-fold and 1.7-fold, respectively, compared with those of the control group (Fig. 4D). Moreover, immunohistochemical staining showed a high level of PPP2R5E in the tumor nodules from the ASO-miR-23a group (Fig. 4E). These data indicated that knocking down miR-23a expression suppressed tumor growth in vivo. Taken together, these results indicated that miR-23a plays a role as an oncogene in gastric cancer.

3.4. PPP2R5E is a target of miR-23a

PPP2R5E is a tumor suppressor and may be a target of miR-23a, which is consistent with miR-23a being regarded as an oncogenic gene. To determine whether miR-23a could regulate the expression of PPP2R5E, we used various algorithms to predict complementary miR-23a-binding sites in the 3′ UTR of PPP2R5E mRNA and determined whether these binding sites were conserved among several species (Fig. 5A). To evaluate whether miR-23a

could directly bind to a target site in the PPP2R5E 3′ UTR, we constructed an enhanced green fluorescent protein reporter vector (EGFP-PPP2R5E 3′ UTR) carrying the PPP2R5E 3′ UTR or a mutant PPP2R5E 3′ UTR (EGFP-PPP2R5E 3′ UTR or EGFP-PPP2R5E 3′ UTR mutant) in which the predicted target regions were cloned downstream of the EGFP stop codon. Additionally, we constructed another EGFP reporter vector (Fig. 5A). pcDNA3 or pri-miR-23a and ASO-NC or ASO-miR-23a were co-transfected into MGC803 cells with the reporter vector (EGFP-PPP2R5E 3′ UTR or EGFP-PPP2R5E 3′ UTR mutant). Compared with the negative control, pri-miR-23a reduced and ASO-miR-23a enhanced the expression of EGFP (Fig. 5B). In contrast, no changes in the intensity of fluorescence driven by the mutant reporter plasmid were observed upon the overexpression or knocked down expression of miR-23a (Fig. 5C). These observations suggested that miR-23a binds to the 3′ UTR of PPP2R5E directly.

Next, we examined the level of endogenous PPP2R5E expression when the level of miR-23a expression was altered. qRT-PCR was performed to assess the expression of PPP2R5E mRNA. When miR-23a was overexpressed, the level of PPP2R5E mRNA was reduced compared with that of the negative group (Fig. 5D). Conversely, compared with that of the ASO-NC groups, the level of PPP2R5E expression in ASO-miR-23a-transfected MGC803 and BGC823 cells was increased (Fig. 5D). Collectively, these results suggested that miR-23a repressed the endogenous expression of PPP2R5E by binding its 3′ UTR directly.

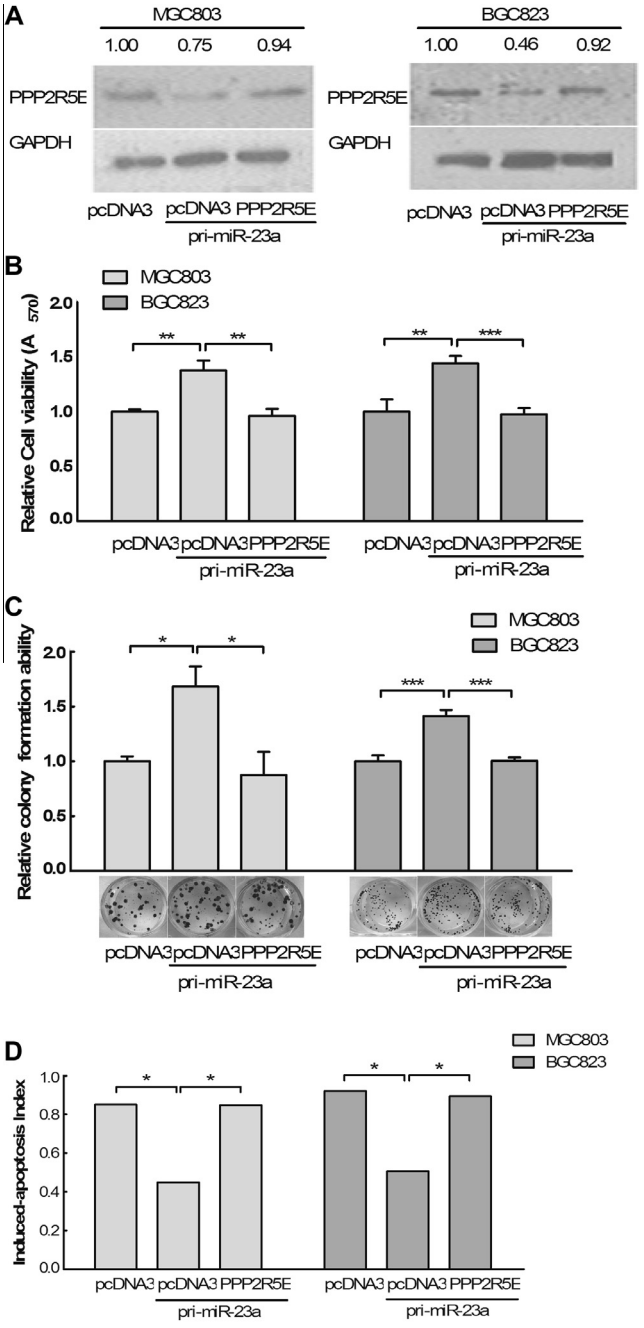

Fig. 6. Restoration of PPP2R5E expression rescued the miR-23a-induced cellular phenotype. MGC803 and BGC823 cells were co-transfected with pri-miR23a and pcDNA3/3flag-PPP2R5E or pcDNA3; the pcDNA3 group served as the control. (A) Western blotting analysis was used to evaluate the expression of PPP2R5E protein. The Arabic numerals above the western blot image indicate the densitometric ratios of PPP2R5E and GAPDH compared with that of the control. (B) Cell viability was determined using an MTT assay 72 h after transfection ($n = 3$, $^{**}P < 0.01$, $^{***}P < 0.001$). (C) Cell clonogenicity was evaluated using a colony-formation assay. The images under the graph show the stained colonies ($n = 3$,$^{*}P < 0.05$, $^{***}P < 0.001$). (D) A TUNEL assay was performed to evaluate apoptosis in the transfected cells. Paclitaxel (0.05ppc) was added to the transfected cells to induce apoptosis. The induced-apoptosis indices are shown ($n = 3$, $^{*}P < 0.05$).

3.5. miR-23a promoted gastric cancer cell growth by downregulating the expression of PPP2R5E

To confirm whether the effects of miR-23a expression on cell proliferation and apoptosis were mediated by PPP2R5E, we overexpressed miR-23a and PPP2R5E in MGC803 and BGC823 cells. Compared with the effect of transfection with the control vector, transfection with pcDNA3/PPP2R5E reversed the negative effects of miR-23a on the expression of PPP2R5E (Fig. 6A). Overexpression of PPP2R5E reversed the increase in cell viability caused by miR-23a (Fig. 6B), and similar results were observed using the colony-formation assay (Fig. 6C). Overexpression of PPP2R5E also neutralized the inhibition of cellular apoptosis that was induced by miR-23a expression, as shown using the TUNEL assay (Fig. S3). Overexpression of PPP2R5E reversed the repressive effects of miR-23a on apoptosis from 45% to 89% in MGC803 cells and from 50% to 90% in BGC823 cells (Fig. 6D) compared with the rates in the control cells. It was noted above that PPP2R5E exerts a tumor-suppressor activity, is directly inhibited by miR-23a and is involved in the miR-23a-mediated malignant phenotype of gastric cancer cells. To summarize, miR-23a plays a growth-stimulating role in gastric cancer cells through at least partially downregulating the expression of PPP2R5E.

4. Discussion

Protein phosphatase 2A (PP2A) is a serine/threonine-specific phosphatase that plays central roles in cellular development, growth and transformation [35]. The role of PP2A as a tumor suppressor that controls tumor progression is thought to be governed by a small subset of specific B subunits that direct PP2A to dephosphorylate certain substrates [36]. The findings of this study support the previously hypothesized role of PP2A as a tumor suppressor gene in breast cancer [34]. In addition, PP2A is involved in cellular transformation as an important tumor suppressor. A large body of evidence suggests that PP2A is a tumor suppressor and plays critical roles in regulating apoptosis [37,38]. Cristobal's group has reported that the inhibition of PP2A is a common event in the genesis of AML and that restoration of the activity of PP2A induces cell-growth arrest and caspase-dependent apoptosis [39].

Herein, we demonstrated that the overexpression of PPP2R5E, a regulatory subunit of PP2A, promoted paclitaxel-induced apoptosis and that knocking down the expression of PPP2R5E inhibited apoptosis in gastric cancer cells. PPP2R5E, which impairs cell prolifera-

tion and induces caspase-dependent apoptosis, was consistently found to affect the activation status of AKT and to reduce the colony-forming ability of leukemic cells [40]. PPP2R5E is also involved in multiple signaling pathways and plays a critical role during early development [41–43]. Moreover, PPP2R5E is an essential regulator of apoptosis and acts as a negative regulator of MAP4K3 by mediating its ability to signal to mTORC1 [44]. PPP2R5E suppresses p53-independent apoptosis during neural development but triggers p53-dependent apoptosis [45]. Recently, Mavrakis et al. found that the oncogenic properties of miR-19b in acute lymphoblastic leukemia were due, at least in part, to its inhibitory effect on PP2A via downregulating the expression of PPP2R5E [38]. Therefore, we hypothesized that, as an important regulatory subunit of PP2A, PPP2R5E acted as a tumor suppressor and played important roles in regulating apoptosis in gastric cancer. Thus, we investigated whether miR-23a regulated the expression of PPP2R5E in the post-transcriptional stage in gastric cancer cells. First, we found that the expression of PPP2R5E was downregulated in gastric cancer cells, and that knocking down the expression of PPP2R5E induced cell-growth inhibition and apoptosis in gastric cancer cells. In our previous study, we used oligonucleotide microarrays to determine that miR-23a expression was upregulated in gastric cancer samples [17]. Thus, we speculated that the downregulation of PPP2R5E expression might be mediated by miR-23a, which might contribute to carcinogenesis. To address this hypothesis, we first used bioinformatics to predict that PPP2R5E is a target gene of miR-23a and then demonstrated an inverse relationship between the upregulation of miR-23a expression and the downregulation of PPP2R5E expression in gastric cancer cells. Furthermore, we determined that the expression of a 3′ PPP2R5E-UTR-based reporter construct was negatively regulated by miR-23a. Overexpressing PPP2R5E was sufficient to counteract the miR-23a-mediated promotion of cellular proliferation and repression of cellular apoptosis. Finally, the results of an animal study suggested that miR-23a promoted the growth of MGC803 tumor cells in vivo. Thus, we have provided evidence that the partial downregulation of PPP2R5E expression by miR-23a may contribute to gastric tumorigenesis.

In summary, the expression of PPP2R5E was suppressed in gastric cancer cells, which promoted the malignant behavior of these cells. The upregulation of miR-23a expression resulted in the downregulation of PPP2R5E expression at the post-transcriptional level, and the anti-proliferative and pro-apoptotic activities of PPP2R5E were negatively regulated by miR-23a, which might provide a partial explanation for the downregulation of PPP2R5E expression in gastric cancer cells.

Competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.05.068.

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